

Engineering Methyllysine Writers and Readers for Allele-Specific Regulation of Protein–Protein Interactions

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Supporting Information

ABSTRACT: Protein–protein interactions mediated by methyllysine are ubiquitous in biological systems. Specific perturbation of such interactions has remained a challenging endeavor. Herein, we describe an allele-specific strategy toward an engineered protein–protein interface orthogonal to the human proteome. We develop a methyltransferase (writer) variant that installs aryllysine moiety on histones that can only be recognized by an engineered chromodomain (reader). We establish biochemical integrity of the engineered interface, provide structural evidence for orthogonality and validate its applicability to identify transcriptional regulators. Our approach provides an unprecedented strategy for specific manipulation of the methyllysine interactome.

Lysine methylation in histones is a post-translational modification that regulates gene expression.¹ This process is catalyzed by lysine methyltransferases (KMTs), classified as “writers”, using S-adenosylmethionine (SAM) (Figure 1).^{2,3}

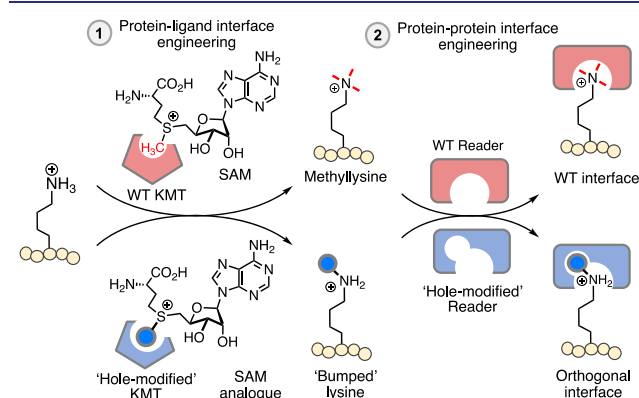


Figure 1. Strategy for allele-specific protein–protein interactions. Methyllysine writer (KMT) is first engineered to introduce “bumped” lysine and disrupt interaction with wild-type readers. Binding is restored using “hole-modified” reader, allowing conditional manipulation of methyllysine pathways.

Biological functions of methyllysine are manifested via specific recognition by conserved protein modules termed “readers” (Figure 1).^{4–6} In humans, an array of ~60 KMTs and >200 proteins with reader domains are involved in establishing and recognizing >5000 methyllysine sites.⁷ Context-dependent function of the methyllysine network is largely unexplored, mainly due to a lack of tools capable of interrogating various

components of the “interactome” simultaneously.^{8–10} Probing how a specific writer–histone–reader axis contributes to chromatin-dependent processes requires precise and sequential perturbation of the methyllysine writers and readers.

Allele-specific chemical genetics (often called the “bump-and-hole” tactic) has emerged as a powerful tool to examine member-specific protein function.^{11–16} We envisioned developing allele-specific writer–histone–reader pairs, fully orthogonal to the human proteome, for precise manipulation of methyllysine interaction (Figure 1). We reasoned a “hole-modified” KMT could accept SAM analogues to modify histone with bulky groups that would perturb the binding of a wild-type reader through steric repulsion. Interaction with the “bumped” histone could be restored with a “hole-modified” reader variant, thus providing control over methyllysine-dependent interactions. Although engineered KMTs are known,¹⁷ evolving a reader to recognize non-native protein modifications is unexplored. We hypothesized that the hydrophobic “aromatic cage” in a reader domain that recognizes methyllysine⁵ could be expanded to accommodate aryllysine in histone and create an orthogonal interface.

We chose trimethylation on H3 at lysine 9 (H3K9me3) as a host system to test the above approach. H3K9 methylation is a marker of transcriptional repression.¹ In humans, a large set of KMTs methylate H3K9, and the modification is “read” by chromodomain and PHD finger containing proteins.^{5,18} Herein, we first develop “hole-modified” H3K9me3 methyltransferases, G9a and Suv39H2, to install lysine modifications that are not recognized by cognate wild-type readers CBX1, 3, and 5; we go on to engineer CBX proteins that bind “bumped” histone and restore the lost interaction. Finally, we provide structural evidence for orthogonality and demonstrate suitability of the engineered system to control a methyllysine-dependent signaling pathway.

To access the “bumped” histone peptides, we synthesized a series of SAM analogues carrying bulky sulfonium alkenyl and aryl groups (Figure 2A, Scheme S1).^{17,19} Each of the SAM derivatives was incubated with previously developed Y1154A mutant of G9a¹⁷ and tetramethylrhodamine (TAMRA) labeled H3K9 peptide. The products were purified and confirmed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Figure S1). The mutant showed complete alkenylation of the substrate (Figure S1); however, it was not efficient at incorporating benzyl modification. We envisioned an aryl group as a lysine modifier would improve binding with

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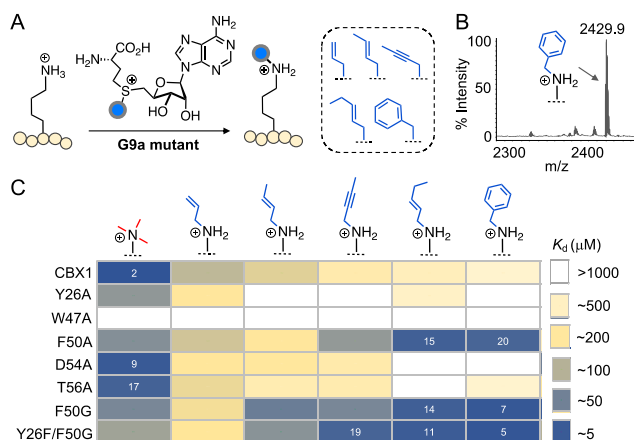


Figure 2. Engineering of G9a and CBX1. (A) G9a-Y1154A mutant transfers alkenyl groups from the corresponding SAM analogues to TAMRA-labeled H3K9 peptide. (B) MALDI-MS showing benzylation of the substrate peptide by G9a-Y1154G mutant using benzyl-SAM. (C) Heat-map diagram showing dissociation constants (K_d) for the binding of modified H3K9 peptides by wild-type CBX1 and “hole-modified” mutants.

engineered CBX by maintaining π – π interaction in the remodeled aromatic cage. We surmised that expanding the pocket of G9a further could facilitate its use of benzyl-SAM. Indeed, Y1154G mutation led to complete substrate benzylation (Figure 2B), demonstrating that G9a can be engineered to accept aryl SAM analogues for expanding the repertoire of “bumped” histone variants. Under our assay conditions, we did not observe any dibenylation of the substrate by G9a mutant (Figure S2).

As a model reader domain for allele-specific engineering, we selected the CBX1 chromodomain, which recognizes H3K9me3 through its aromatic cage.²⁰ First, using a fluorescence polarization assay, we determined the dissociation constants (K_d) of wild-type CBX1 chromodomain from TAMRA-labeled H3K9me3 and various “bumped” variants (Figure 2C, S3). The reader protein bound H3K9me3 tightly, with a K_d ($2.0 \pm 0.1 \mu\text{M}$) close to the reported value.²¹ CBX1 showed 53-fold weaker binding affinity ($K_d = 106 \pm 20 \mu\text{M}$) toward allyl-modified peptide. Importantly, it failed to bind peptides with modifications bulkier than allyl ($K_d > 500 \mu\text{M}$, Figure S3), presumably due to a steric clash inside the aromatic cage.

In order to develop an orthogonal reader, we analyzed a published structure of the CBX1 chromodomain and identified residues Y26, W47, F50, D54 and T56 that recognize H3K9me3.²² We generated the corresponding alanine mutants and measured their binding affinity toward all the modified peptides (Figure 2C, S4–8). Compared to wild-type CBX1, the mutants showed decreased binding toward the trimethylated peptide, indicating that an intact hydrophobic pocket is essential for optimum recognition of H3K9me3.²¹ Among the CBX1 mutants examined, F50A showed significant gain in binding affinity toward the pentenyl and benzyl peptides (K_d of 14.1 ± 2.8 and $18.5 \pm 4.5 \mu\text{M}$, respectively), while affinity for H3K9me3 decreased by almost 32-fold (K_d of $63.5 \pm 6.2 \mu\text{M}$) compared to wild-type reader (Figure 2C, S6). Encouraged by this result, we prepared F50G mutant with a larger cavity in an effort to further improve the binding efficiency. F50G indeed showed a 3-fold improvement in affinity toward H3K9bn (Figure 2C, S9).

To improve orthogonality of the engineered readers, we generated several double mutants by combining F50G with mutations at Y26. Most mutations led to complete loss of binding (Figure S10–12); however, a Y26F/F50G variant with intact aromatic side chain at F26 maintained robust affinity toward benzyl peptide ($K_d = 5.5 \pm 0.9 \mu\text{M}$; Figure 2C, S10). Importantly, interaction with H3K9me3 peptide was reduced to a K_d of $\sim 80 \mu\text{M}$. We further confirmed the binding constants by isothermal titration calorimetry using H3K9me3 and benzylated (H3K9bn) peptides. Both native and engineered readers showed comparable affinity to their cognate peptides (Figure 3A,B, S13, Table S1). Furthermore, when

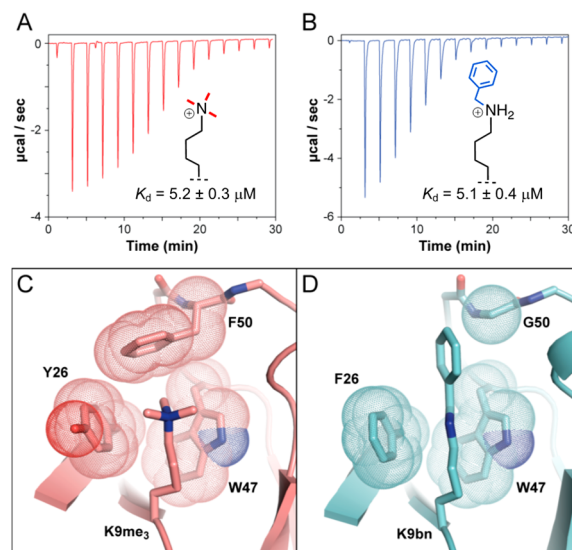


Figure 3. Characterization of the histone-reader pairs. (A, B) Isothermal titration calorimetric (ITC) measurements for binding between wild-type CBX1 and H3K9me3 (A), and Y26F/F50G-CBX1 and H3K9bn (B). (C, D) View of the aromatic cage from crystal structures of wild-type CBX1 chromodomain bound to H3K9me3 peptide (C) and “hole-modified” CBX1 (Y26F/F50G) bound to H3K9bn (D).

tested against the H3K9me2 peptide, a major enzymatic product of G9a, wildtype and engineered CBX1 bound with K_d of $18.3 \mu\text{M}$ and $>300 \mu\text{M}$, respectively (Figure S14). Taken together, our mutational study led to an orthogonal chromodomain capable of recognizing “bumped” histone peptide—a new approach to modulate histone-reader interactions.

To gain further insight into the orthogonality of the bump–hole pair, we grew crystals of the complex of native CBX1 with H3K9me3 as well as the complex of Y26F/F50G-CBX1 with H3K9bn by hanging drop vapor diffusion. We collected diffraction data on each complex, solved the native structure (PDB 6D07) by molecular replacement with a model derived from a structure of CBX5 bound to H3K9me3,²¹ and solved the mutant structure (PDB 6D08) using the refined model of the native CBX1 complex. Both structures were refined to 2.1 Å resolution (Figure 3C,D, Table S2). The overall fold and binding interactions observed for native CBX1 in complex with H3K9me3 are identical to those noted in closely related members CBX3 and CBX5 (Figure S15).²¹ The backbone coordinates for the bump–hole mutant complex were virtually identical to native CBX1 (0.6 Å C_α rmsd, Figure S15). Inspection of the aromatic binding pocket shows that the

benzyl “bump” effectively fills the hole created by the F50G mutation (Figure 3D) and that removal of the side chain in CBX1 has no measurable effect on local backbone conformation. Superimposition of the two complexes revealed that F50 of wild-type CBX1 undergoes steric interference with benzyl group of H3K9bn (Figure S15), explaining the observed orthogonality.

Sequence analysis of the major KMTs revealed that Y1154 in G9a is highly conserved among writers (Figure 4A) and an

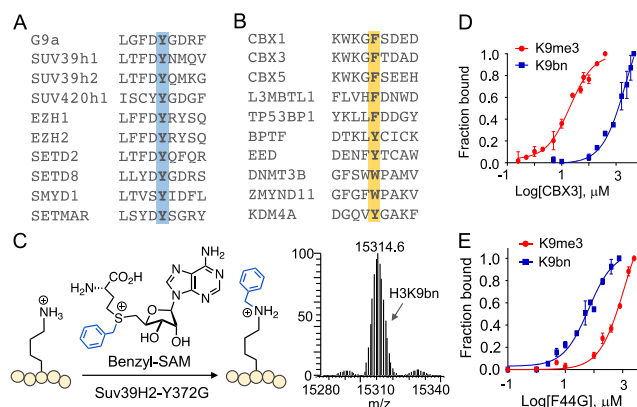


Figure 4. Generality in methyllysine writer-reader engineering. (A, B) Sequence alignments of KMTs (A) and methyllysine readers (B) showing Y1154 of G9a and F50 in CBX1 are highly conserved. (C) Substrate benzylation by Suv39H2-Y372G mutant on full-length histone H3. (D) Binding of wild-type CBX3 toward H3K9me3 ($K_d = 12.2 \pm 0.2 \mu\text{M}$) and H3K9bn ($K_d > 1 \text{ mM}$). (E) Binding of CBX3-F44G mutant toward H3K9me3 ($K_d > 1 \text{ mM}$) and H3K9bn ($K_d = 49 \pm 2.4 \mu\text{M}$).

aromatic residue, equivalent to F50 in CBX1, is present in all of the readers analyzed (Figure 4B).²³ These bulky amino acids are acting as gatekeepers that preclude binding of SAM and methyllysine analogues to writers and readers, respectively. We focused on another H3K9me3 methyltransferase, Suv39H2, and the cognate reader CBX3.^{24,25} The hole-modified Y372G mutant of Suv39H2, equivalent to G9a-Y1154G, efficiently utilized benzyl-SAM to modify substrate peptide as well as full-length H3 (Figure 4C, S16, S17).

To demonstrate generality among reader domains, we prepared an F44G mutant of CBX3, equivalent to Y26F/F50G-CBX1. Conveniently, CBX3 already carries a phenylalanine at the position equivalent to Y26 in CBX1. Wild-type CBX3 recognized H3K9me3 peptide effectively as judged by fluorescence anisotropy, but no binding was observed toward the benzylated homologue (Figure 4D). In contrast, the “hole-modified” CBX3 exhibited enhanced affinity for H3K9bn with significant loss of binding toward the trimethyl mark (Figure 4E). These results establish that multiple histone writers and readers can be engineered at gatekeeper sites to access orthogonal protein–protein interfaces.

To determine if the engineered writer and reader could act in tandem, we first benzylated full-length H3 and purified mononucleosomes using the Suv39H2 mutant followed by enrichment with the hole-modified CBX1. We observed a signal for H3 only in the presence of benzyl-SAM as evident from Western blot data (Figure S18), demonstrating successful reading and writing of H3K9bn on full-length substrates.

We further investigated whether the orthogonal pairs are functional in a cellular milieu (Figure 5A, S19). HEK293T cells

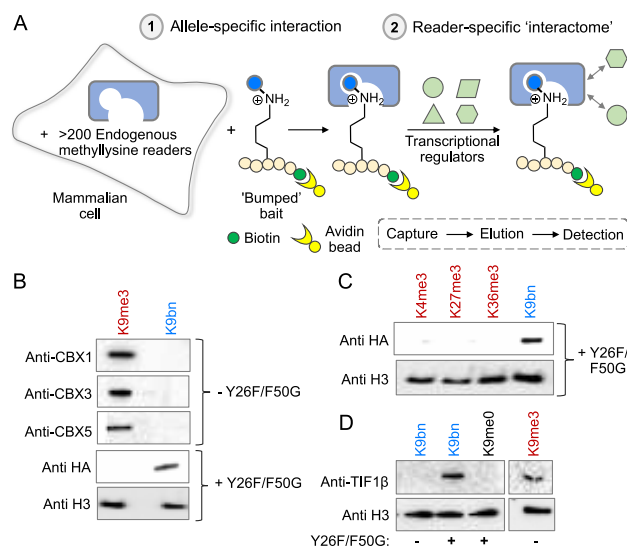


Figure 5. Allele-specific interactions demonstrated in cell extracts. (A) Interaction between “hole-modified” CBX1 and “bumped” histone in the presence of endogenous methyllysine readers (step 1). The engineered complex binds to transcriptional regulators (step 2). (B) Western blot (indicated antibody) of nuclear extracts pulled-down using biotin-attached H3K9me3 or H3K9bn peptide. CBX1 mutant carries HA tag. (C) Western blot (anti-HA antibody) of nuclear extracts pulled-down using biotin-attached H3K4me3, H3K27me3, H3K36me3 or H3K9bn peptide. (D) Western blot (anti-TIF1β antibody) of nuclear extracts pulled-down using biotin-attached H3K9me3 or H3K9bn or unmodified peptide.

were cultured to express full-length hole-modified Suv39H2 variant, and the isolated nuclei were incubated with benzyl-SAM in a pulldown assay. Only the benzylated H3 was found to be specifically enriched with the CBX1 mutant (Figure S19), indicating that the engineered pair could act on chromosomal histone. Next, we expressed a full-length CBX1 variant carrying the engineered chromodomain.²⁶ Nuclear extracts were incubated with biotinylated histone peptides, each carrying either unmodified, methylated or benzylated lysine, pulled down with avidin beads and analyzed using antibodies for CBX1, 3, or 5 (Figure 5B, S20). While the trimethylated peptide enriched all three wild-type CBX proteins, the benzylated bait was specific for hole-modified CBX1 (Figure 5B, S21). Furthermore, biotinylated peptides carrying methyllysine at a site different from H3K9 failed to interact with the mutant (Figure 5C, S21), confirming competency of the engineered system to mediate allele-specific histone–reader interactions. As we only examined CBX1, 3, and 5, detailed proteomic analysis of enriched proteins will be required to evaluate specificity of H3K9bn across all the methyllysine readers.

Finally, we examined interactions between the CBX1 variant and transcriptional complexes (Figure 5A). Binding of full-length CBX proteins to H3K9me3 creates a docking site for transcriptional regulators such as TIF1β.^{26,27} We incubated biotinylated H3K9bn peptide with nuclear extract of HEK293T cells expressing the “hole-modified” reader, and enriched the “interactome” using avidin and analyzed by Western blotting with TIF1β antibody (Figure 5D, S21). We observed selective pull-down of TIF1β by benzylated peptide, but not the unmodified peptide, only when cells expressed the “hole-modified” CBX1 mutant. The “bumped” peptide failed to interact with transcriptional regulators without the

engineered reader. As a positive control, trimethylated peptide was shown to enrich TIF1 β using endogenous CBX proteins. This set of results demonstrated that the engineered system maintains native-like interactions between a specific methyl-lysine reader and transcriptional regulators.

In summary, we offer a strategy to modulate protein–protein interactions particularly those governed by lysine methylation. Development of orthogonal pairs for protein–protein interactions is rarely attempted.^{28–30} Using a set of H3K9me3 writers and readers as a paradigm, we showed that the remodeled interface is orthogonal to the wild type. We established biochemical integrity of the engineered interface, provided structural rationale for orthogonality, demonstrated generality of the approach, and validated functional compatibility of the synthetic interface in recognizing transcriptional regulators. We anticipate developing an *in cellulo* assay to synthesize benzyl-SAM by a promiscuous SAM synthetase mutant using benzyl methionine precursor.³¹ Such an engineered benzyllysine apparatus would allow installing the unique modification on chromosomal histone to interrogate a specific methyllysine pathway within cell for orthogonal manipulation of mammalian gene expression.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b05725.

Methods for protein expression and crystallization, biochemical assays, supplementary figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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